SHORT COMMUNICATION

Diazotrophy within *Desulfovibrio*

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Use of a pyruvate-based medium in which the supply of combined N limited growth enabled detection of diazotrophy (as C_2H_2 reduction) in batch cultures of 12 out of 15 strains of *Desulfovibrio* representing 5 species and including 2 strains hitherto believed to be non-diazotrophic.

INTRODUCTION

Nitrogen fixation among *desulfovibrios* has been subject to doubts (Postgate, 1984), which arose principally from capriciousness of diazotrophic growth within this group and low levels of nitrogenase activity. Postgate & Kent (1984) showed that two conditions were necessary for derepression of nitrogenase in *Desulfovibrio gigas*: the population needed to be N-limited before exposure to derepressing conditions and exogenous sodium pyruvate and sulphate were required during derepression. Our studies on lactate-grown N-limited populations from a chemostat prompted us to seek a N-limiting pyruvate-based medium for derepression of nitrogen fixation in batch cultures. Some of this work was briefly reported by Postgate *et al.* (1985).

METHODS

Organisms, growth and nitrogenase assays. *Desulfovibrio* strains (Table 1) were obtained from the NCIMB except for *D. gigas* (a gift from Dr E. C. Hatchikian, CNRS, Marseille, France) and *D. thermophilus* (a gift from Dr F. Widdel, University of Konstanz, FRG) and were checked for aerobic and anaerobic contaminants (Postgate, 1984). Stocks were maintained in anaerobic lactate-based medium B of Postgate (1984) and working stocks subcultured in his lactate-based medium C, both supplemented with 2.5% (w/v) NaCl for strains of marine or brackish origin. Derepression of nitrogenase activity was tested by growing cultures in 10 ml low-N pyruvate media (see below), using a 1:20 inoculum to ensure acceptably low carry-over of fixed N, in test tubes flushed aseptically with O_2 -free N_2 and sealed with Suba-seal rubber closures. Cultures were incubated until maximum turbidity was reached as judged by eye (2–6 d). The incubation temperature was 29 °C except for *D. thermophilus*, which was grown at 65 °C. Samples (1 ml) were transferred aseptically with minimal exposure to O_2 to Suba-sealed 7 ml ampoules; these had been previously flushed with N_2 and contained 0.1 ml of the derepression medium to provide extra substrate for derepression. C_2H_2 (0.7 ml) was injected and C_2H_4 production at 28 °C was assayed by gas chromatography at intervals over 2–4 h. *D. thermophilus* was assayed at both 28 °C and 65 °C.

Populations grown with 0.1% (w/v) NH_4Cl were included at least once with each strain as controls to demonstrate NH_4^+ repression and to exclude non-diazotrophic C_2H_4 formation.

Media. Medium PYR contained ($g\ l^{-1}$): K_2HPO_4 , 0.5; $CaCl_2 \cdot 2H_2O$, 0.1; $MgCl_2 \cdot 6H_2O$, 1.6; $FeSO_4 \cdot 7H_2O$, 0.012; Na_2MoO_4 , 0.001; sodium pyruvate, 10; Na_2SO_4 , 12; NaCl, 25 (for salt-requiring strains only). The medium was sterilized and stored; shortly before use, cysteine hydrochloride ($0.15\ g\ l^{-1}$) was added to a portion and the pH adjusted to 7.6 ± 0.1 . It was steamed for 10 min, distributed while hot and used as soon as possible. Medium PYR-CAA was PYR plus Difco Casamino acids, $0.1\ g\ l^{-1}$. Medium PYR-PAA was similar but Casamino acids were replaced by ($g\ l^{-1}$) proline, 120, arginine, 40 and neutralized aspartic acid, 80, added aseptically after steaming and adjustment of the pH of the basal medium.

Table 1. C_2H_2 reduction in *N*-limited cultures of *Desulfovibrio*

Species	Strain designation*	Habit†	Positive C_2H_2 reduction/ no. of tests		Maximum activity [nmol C_2H_2 min ⁻¹ (mg protein) ⁻¹]	
			Medium:		Medium:	
			PYR-CAA	PYR-PAA	PYR-CAA	PYR-PAA
<i>D. gigas</i>	—	F	3/3	1/1	2.2	
<i>D. vulgaris</i>	Hildenborough	F	1/4	2/2		1
	Wandle	F	0/3	2/2		2.4
	Groningen	F	2/3	1/1	8.8	
	Monticello 2	F	3/3	2/2	4.5	4.5
<i>D. desulfuricans</i>	Berre sol	F	2/2	1/1	0.7	
	Norway 4	S	2/4	1/1	1.4	
	Canet 41	S	0/3	0/4	0	0
	El Agheila Z	S	1/1	1/1	15.3	
	Teddington R	F	2/2	1/1	5	
<i>D. salexigens</i>	British Guiana	S	3/3	1/1	9.5	
	California 43:63	S	1/1	1/1	2	2
<i>D. africanus</i>	Benghazi	F	2/3	1/3	1.2	
	Walvis Bay	S	0/3	0/8	0	0
<i>D. thermophilus</i>	—	F	0/9‡	0/5‡	0	0

* For NCIMB numbers see Table 2.

† F, fresh water; S, saline.

‡ 5 tests at 29 °C, 4 at 65 °C in PYR-CAA; 3 at 29 °C, 2 at 65 °C in PYR-PAA.

RESULTS

Cultures in medium PYR-CAA showed positive C_2H_2 reduction by 11 of the 15 strains examined, but 4 of the positive strains sometimes gave negative results (Table 1). Cultures in medium PYR-PAA behaved more consistently, except in the case of strain Benghazi, and diazotrophy in strain Wandle was detected only in this medium. Strains Benghazi and British Guiana were earlier recorded as non-diazotrophic (Postgate, 1970). None of the strains tested showed acetylene reduction if NH_4Cl was present.

Cultures of the mesophiles in either pyruvate medium developed a light pink colour which was not due to a fluorescent product nor to an excreted rubredoxin or cytochrome (data not shown).

D. thermophilus and strains Walvis Bay and Canet 41 were the only strains consistently negative for C_2H_2 reduction. We therefore undertook a more thorough examination of strain Canet 41. An NH_4^+ -limited chemostat culture growing in lactate medium was set up as described by Postgate & Kent (1984) for *D. gigas*, except that the medium contained 2.5% (w/v) NaCl. Samples from steady states at $D = 0.055$, 29 °C, were centrifuged aseptically and allowed to derepress in medium PYR, PYR-CAA, PYR-PAA or in PYR with either glutamate or aspartate (100 μg ml⁻¹), all the media containing 2.5% (w/v) NaCl. Since a nitrogenase system repressed by Mo is known to exist (Bishop *et al.*, 1980), medium PYR chemically freed of Mo and with Fe restored was also tested for support of C_2H_2 reduction with and without Casamino acids. All conditions were tested twice, with chemostat populations taken on different days, some (PYR-CAA, PYR-PAA, PYR + aspartate) with three such populations. No derepression of nitrogenase was detected after 24, 48 and, in one test, 72 h by C_2H_2 reduction assays lasting 24 h. Samples of the chemostat culture were twice used as inocula for PYR-CAA and PYR-PAA for four of the tests recorded in Table 1. A sample of the lactate-sufficient, NH_4^+ -limited chemostat population was also assayed directly with C_2H_2 over 24 h with the expected negative result. We conclude that strain Canet 41 must be regarded as phenotypically Nif^- .

DISCUSSION

The failure of three strains to show C_2H_2 reduction in our hands may be an extreme example of the capriciousness shown by, for example, strain Benghazi with both PYR-CAA and PYR-PAA. The three amino acids in PYR-PAA were chosen because regulation of their utilization in

Table 2. Collected reports of diazotrophy in *Desulfovibrio* species

Species	Strain designation	Strain no.	Reference*					
			1	2	3	4	5	6
<i>D. gigas</i>	—	NCIMB 9332	(+)	+	+			+
<i>D. vulgaris</i>	Hildenborough	NCIMB 8303		+				+
	Wandle	NCIMB 8305						+
	Groningen	NCIMB 11779			+			+
	Monticello 2	NCIMB 9442						+
	—	—				+		
<i>D. desulfuricans</i>	Berre sol	NCIMB 8388	+					+
	Berre eau	NCIMB 8387	+					
	Norway 4	NCIMB 8310		+	+			+
	Canet 41	NCIMB 8393						—
	El Agheila Z	NCIMB 8318						+
	Teddington R	NCIMB 8312						+
	Essex 6	NCIMB 8307			+			
	El Agheila A	—			+			
	—	2198					(+)	
<i>D. salexigens</i>	British Guiana	NCIMB 8403			—			+
	California 43:63	NCIMB 8364						+
<i>D. africanus</i>	Benghazi	NCIMB 8401			—			+
	Walvis Bay	NCIMB 8397			—			—
	—	2372					+	
<i>D. baculatus</i>	X	—					+	
<i>D. thermophilus</i>	—	7†					(+)	—

* References: 1, Le Gall *et al.* (1959); 2, Riederer-Henderson & Wilson (1970); 3, Postgate (1970); 4, Sekiguchi *et al.* (1977); 5, Nazina *et al.* (1979); 6, this report. (+) signifies an ambiguous or tentative report.

†DSM number 1276, USSR collection number VKM V-1128.

Klebsiella pneumoniae requires at least part of the regulatory apparatus of *nif* to be functioning (see Postgate, 1982) and though it increased the probability of demonstrating C_2H_2 reduction, we may still not have found the precise requirement for C_2H_2 reduction by these strains. However, in the light of our examination of strain Canet 41 we believe this situation is unlikely.

Table 2 collects all published data on diazotrophy in *Desulfovibrio*; all tests except ours were made in lactate media. Our tests add seven to the number of strains which show diazotrophy. Nazina *et al.* (1979) obtained 'good' C_2H_2 reduction from *D. africanus* 2372 and *D. baculatus* but activities from *D. desulfuricans* 2198 and *D. thermophilus* were very slight and similar; their lack of NH_4^+ -repressed controls added an element of doubt about the significance of these low values. In 16 tests we found no activity at all with our cultures of *D. thermophilus* strain 7; these included two tests in the lactate-based medium used by Postgate (1970). Thus out of 22 published pure strains of *Desulfovibrio* examined, 18 showed clear evidence of diazotrophy, covering six species including the representative of *D. baculatus*.

Our findings demonstrate that absence of growth and C_2H_2 reduction in a lactate medium with low or zero combined N is not an adequate criterion of diazotrophy among desulfovibrios. Other diazotrophs may also require special cultural conditions in addition to deprivation of fixed N to express their diazotrophy.

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